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(U) STRUCTURE AND DESIGN OF MULTIPOTENT PEPTIDE MICROBICIDES

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A family of antimicrobial leukocyte peptides has been isolated from the neutrophils of several species. I am using the concensus structure of the peptides (known as defensins) as a molecular foundation for generating new antimicrobial peptides by synthetic methods. The synthetic approach is directed by correlating the solution structures of various defensins with their distinctive biological activities.

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Structure and Design of Multipotent Peptide Microbicides

ONR-N00014-86-K-0525
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ANNUAL REPORT

Project goal. The goal of this project is to design novel peptide antibiotics using a naturally occurring family of peptides, known as defensins, as models. Defensins are homologous peptides, 29-34 residues in length, which are major constituents of the cytoplasmic granules of polymorphonuclear leukocytes. The structural hallmark of the defensin peptide family is a conserved infrastructure comprised of 1 arginine, 1 glycine, and 6 disulfide-linked cysteine residues (Fig. 1). Although the peptides are similar in their overall fold, they possess diverse antimicrobial spectra and potencies. By correlating specific biocidal activities with unique structural features, we seek to design custom peptide antimicrobials based on structure-function principles derived from these studies. Insight into the molecular details of the peptide-target cell interactions may contribute to the general understanding of protein-membrane recognition processes.

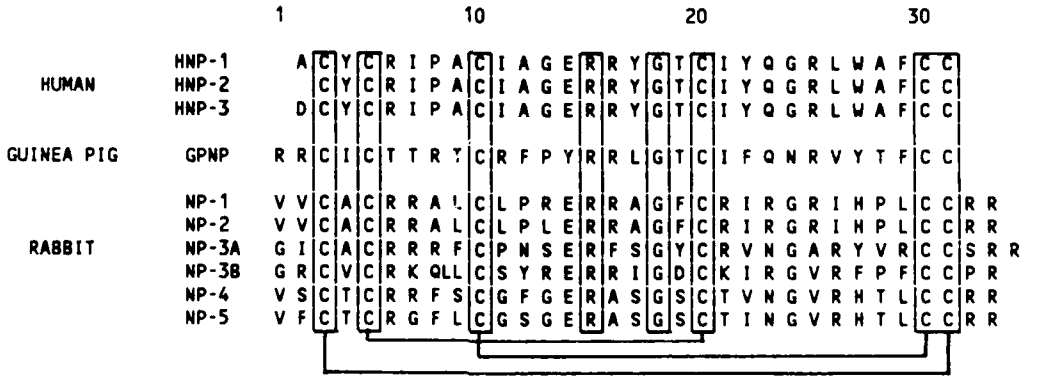

Figure 1. Covalent structures of defensins. The single letter amino acid code for ten defensin peptides from the indicated sources are shown maximally aligned. Invariant residues are outlined. The cysteine connectivities are also indicated.

Recent Progress:

1. **Isolation of new members of the defensin family**. Using methods established for the identification, purification, and characterization of rabbit, human, and guinea pig defensins, we isolated three new members of this peptide family from rat peritoneal neutrophils. The new members of the defensin family retain the conserved (6 Cys, 1 Gly, 1 Arg) infrastructure, are 29-32 residues in length, and have similar broad spectrum antimicrobial activities as many of the previously characterized defensins. Comparison of their structures and biocidal activities will be used for correlating residue-specific antimicrobial function(s).

2. **Chemical modifications of defensins**. By comparing the antimicrobial spectra of various members of the defensin family, we found that HNP-1 and HNP-3 have distinctly different potency against fungal test organisms (Candida albicans) even though these twp defensins vary in sequence by a single residue (Fig. 1). This suggested that the amino terminal portion of the peptide plays an important role in function. To probe the relationships between the structure and function at this molecular locus, we have evaluated the effects of various covalent modifications of the amino terminus of HNP-1. We have developed methods for specific modification, purification, and characterization of Ala_1 derivatives of HNP-1 using fluoroscein isothiocyanate, succinic anhydride, ethylacetimidate, and cyanate. While the characterization of the activities of these HNP-1 derivatives is not yet complete, our preliminary studies indicate that fluorescenation (conversion to a neutral amino terminus) abolishes HNP-1 activity, and amidination (with retention of native amino terminal charge) results in a compound with activity equal to that of the unmodified peptide. We are likewise generating defensin derivatives modified at the C-terminus using carbodiimide-mediated coupling of taurine, glycinamide, and arginine amide.

3. **Solution Structures**. In collaborative studies performed with Arthur Pardi, we have determined the solution structures of four defensin peptides: human defensins HNP-1 and

HNP-3, and rabbit defensins NP-2 and NP-5. The solution structures were determined by 2-D NMR experiments (see list of publications) and refined by superimposing the disulfide array which we determined by a novel method. As anticipated, the four defensins studied by this technique have very similar structures. A space filling model of HNP-1 is shown in Figure 2, demonstrating that this defensin possesses an amphiphilic topology.

Figure 2 Space filling model of HNP-1 derived from NMR spectroscopy and covalent structure determination. Main chain atoms are shown in white, charged residues are shown in red, weakly polar residues are shown in blue, and hydrophobic residues are green or yellow (cystines).

One face of the peptide is comprised of hydrophobic residues, while the charged R groups are segregated, and are substantially clustered. The folded configuration is unique among peptides of known structure, and it varies in particular from amphiphilic alpha helical structures which have been described. The significance of the amphiphilic array is discussed below.

4. **Membrane active features of defensins**. A number of experimental observations regarding the range of defensin bioactivities suggested that they act at the level of the target membrane: the peptides are active against prokaryotes, eukaryotes, and enveloped (but not naked) viruses; susceptible targets are protected by temperatures below the phase-transition point of the target membranes; bacteria and fungi are rendered insensitive to defensins by proton ionophores such as DNP or CCCP. We have now shown that at least two defensins, HNP-1 and NP-1, form voltage-dependent channels in lipid bilayer membranes. The pores formed 1) require specific orientation of the voltage potential, 2) are anion selective, 3) are heterogeneous, and 4) appear to require the association of 2-4 monomers in the bilayer. These features are consistent with a novel oligomeric membrane channel which likely contributes to the biocidal mechanisms of defensin peptides.

5. **Synthetic Defensins**. We previously reported the synthesis of the rabbit defensin NP-2 using t-BOC chemistry and HF cleavage. The yield by this method was approximately 5%. We have modified the protocol used for synthesis and refolding such that our yields are now ca. 22%. This was accomplished by using TFMSA cleavage and modified conditions for peptide refolding and disulfide oxidation. With the knowledge gained from development of NP-2 synthesis conditions, we have succeeded in synthesizing more than 200 mg of the human defensin HNP-1. This peptide contains 30 residues, which include 3 tryrosines, 1 tryptophan, and 6 cysteines. A manuscript describing the conditions used for synthesis, cleavage, deprotection, refolding, and biologic activities of synthetic HNP-1 is in preparation. The ability to synthesize the quantities of this and related defensins will allow us to perform material-consuming studies which would otherwise be impossible. Further, we are now well positioned for the synthesis of defensin congeners, a major goal of this project.

6. **Crystallographic studies**. Ongoing analysis of diffraction quality crystals of the rabbit defensin NP-2 and the human defensin HNP-1 have proceeded to the point of tracing the

main chain of both peptides. While both NP-2 and HNP-1 form high quality crystals, we have had difficulty in generating heavy atom derivatives for isomorphous replacement studies. In order to increase the number of possible crystal forms amenable to heavy atom derivatives, we sought to crystallize the human defensin HNP-3. With its amino terminal aspartate, we surmised that a heavy cation might bind to this site. High grade crystals HNP-3 were recently grown at pH 4.05 from a precipitant of sodium citrate, ethanol and polyethylene glycol (Fig. 3). We are currently characterizing the crystal space group, and will collect native data sets shortly.

Figure 3 10X photo of crystals of HNP-3

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